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Determination of Vitamin A Ester in Fortified Poultry Mashes

With Activated Glycerol Dichlorohydrin

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A study has been made of the application of the vitamin A–activated glycerol dichlorohydrin reaction to the estimation of vitamin A in fortified poultry mash. Certain constituents present in both unsaponified and saponified petroleum ether feed extracts repress the vitamin A–glycerol dichlorohydrin reaction. Adsorption on a mixture of 3.1 Hyflo Super-cel–activated magnesia No. 2641 removed most of the interfering substances. By carefully regulating the length of the adsorption column, volume of eluant, and quantity of sample adsorbed, it was possible to elute vitamin A ester while retaining carotene and most unknown interfering compounds on the column. With most feeds, vitamin A concentrations of 3000 or more I.U. per pound could be accurately determined within ±5%, and concentrations as low as 1500 I.U. per pound could be determined with accuracy sufficient for routine purposes, except when the mash contained a large proportion of fish meal. Quantities as low as 500 I.U. per pound could not be accurately determined. Chief disadvantages: It is necessary to use an empirical correction formula and the method is not applicable to free vitamin A alcohol.

SOBEL and Werbin have recently published papers (*6*, *7*) on a new method for the colorimetric determination of vitamin A in which activated glycerol dichlorohydrin is used as the color-producing agent. Because the color formed is relatively stable and with fish liver oils the glycerol dichlorohydrin reaction gives results in close agreement with the antimony trichloride and ultraviolet absorption methods, these workers propose the adoption of glycerol dichlorohydrin in preference to antimony trichloride as the color-producing agent (*7*).

The authors attempted to apply the glycerol dichlorohydrin reaction to petroleum ether (boiling point, 63° to 70° C.) extracts of fortified poultry mash. (All the petroleum ether used in this investigation had a boiling point range of 63° to 70° C. and received no special purification treatment.) In preliminary experiments with both unsaponified and saponified samples, inability to recover more than a small fraction of added vitamin A ester indicated that certain constituents in both the unsaponified and saponified extracts suppressed or interfered with the glycerol dichlorohydrin reaction with vitamin A. Brew and Scott (*1*) and Cooley *et al.* (*2*) have shown that constituents of certain feed extracts give false vitamin A color reactions with antimony trichloride. The problem with the glycerol dichlorohydrin reaction is somewhat different in that the chief effect of interfering substances seems to be a suppression of the glycerol dichlorohydrin–vitamin A reaction.

A chromatographic study was made with a number of adsorbents in an attempt to find one that would not only remove the substances that interfered with the glycerol dichlorohydrin–amin A reaction but would also separate vitamin A ester from any carotenoids present, so that a correction for the presence of carotenoids could be avoided. It was found that under carefully controlled conditions an adsorbent consisting of 3 parts of Hyflo Super-cel and 1 part of activated magnesia No. 2641 would meet most of the requirements. It was impossible to remove all the substances in various feed extracts which repress the glycerol dichlorohydrin–vitamin A reaction. An empirical correction formula was developed that permitted reasonably good estimation of vitamin A ester in a variety of poultry mashes and at concentrations as low as 1500 I.U. per pound.

METHOD

Extraction and Purification. All manipulations are carried out in a semidarkened room shielded from direct sunlight, with all-glass apparatus. Ten to 20 grams of mash are extracted with petroleum ether for 3 hours in a Soxhlet extractor. For best results the sample should have a minimum of 6 to 8 I.U. per gram, although feeds containing half this quantity can be analyzed with reasonable accuracy.

The extract is concentrated under vacuum and mild heating, transferred to a 50-ml. volumetric flask, and made to volume with the petroleum ether. Aliquots equivalent to 4 to 5 grams of original sample are taken for purification by adsorption. The adsorbent is a mixture of 3 parts of Hyflo Super-cel (Johns-Manville Company) and 1 part of activated magnesia No. 2641 (Westvaco Chemical Corp.). The components are mixed in a ball mill (stones are omitted). A 7 × 2.5 cm. column of adsorbent is prepared by firmly tamping the dry adsorbent in a suitable tube under full vacuum. Details of this technique have been reported (*9*).

The column is wet with petroleum ether followed immediately by the aliquot. The vitamin A ester is eluted with 25 ml. of 5% acetone in petroleum ether. The flow of solvent through the column is regulated so that a rapid drop rate is maintained.

In order to remove fine adsorbent particles, the eluate is filtered on a small pad of Hyflo Super-cel in a sintered Hirsch funnel, which is then washed several times with petroleum ether. The filtrate is concentrated, and transferred to a 50-ml. standard taper Erlenmeyer flask, and the solvent is evaporated to dryness with vacuum and mild heating. The residue is dissolved in 1 ml. of chloroform.

To a duplicate aliquot is added a suitable quantity of standard vitamin A ester. Twenty International Units were used in this investigation, and the fortified sample was adsorbed and eluted in the manner described.

Colorimetric Determination. A calibration curve of International Units of vitamin A against density in the range 0 to 50 I.U., is prepared according to the method of Sobel and Werbin (*6*); a standard concentrate of vitamin A ester, capsules PC-3, from Distillation Products Company, is used as the source of vitamin A. To the vitamin A in 1 ml. of chloroform are added 4 ml. of activated glycerol dichlorohydrin (Shohan Laboratories), the mixture is shaken vigorously, and the density is determined 2 minutes after mixing. The authors used a Fisher electrophotometer provided with microtubes and a 550-mμ filter. A blank consisting of 1 ml. of chloroform and 4 ml. of glycerol dichlorohydrin is used for the zero setting of the instrument. The calibration curve secured with the Fisher electrophotometer was not linear, and it was necessary to make use of the standard reference curve in all calculations.

The density of the purified mash solutions is determined in the same manner as that of the standards. In calculating the results it is necessary to allow for the repression of the vitamin A–glycerol dichlorohydrin reaction which occurs to some extent even in extracts purified by adsorption. Vitamin A in the aliquot taken can be calculated by the following formula:

$$\text{I.U. of vitamin A} = \left(\frac{X + Y}{Z}\right) \times X$$

in which X = I.U. of vitamin A found in unfortified aliquot, Y = I.U. of standard vitamin A added to fortified aliquot, and Z = I.U. of vitamin A found in fortified aliquot.

The expression $\left(\frac{X + Y}{Z}\right)$ represents an empirical correction factor which usually had a value of 1.05 to 1.15 when an aliquot equivalent to 4 to 5 grams was taken for analysis. The reason for using this factor instead of the repression formula $\left(\frac{Y}{Z - X}\right)$ developed by Oser, Melnick, and Poder (*4*) is discussed below.

Table I. Effect of Size of Sample on Repression Factor

(40 units vitamin A ester added to sample in all cases)

Sample, Grams	Density	Repression Factor
0	0.265	1.0
4.0	0.241	1.1
7.0	0.204	1.3
8.0	0.189	1.4
10.0	0.177	1.5
16.0	Impossible to read because of turbidity	...

DISCUSSION AND RESULTS

In order to determine vitamin A in poultry mashes with the glycerol dichlorohydrin reaction, it is necessary to separate it from many interfering substances present in the original extract. This separation is based on a critical relationship among the quantity of adsorbent, volume of eluant, and amount of sample taken for analysis. Standard solutions of vitamin A ester equivalent to 10 to 100 I.U. could be recovered within ±5% when a firmly tamped magnesia adsorbent, 7 × 2.5 cm., and 25 ml. 5% acetone in petroleum ether eluant were used. When longer columns were tested, considerable vitamin A was lost even when much more eluant was used. These results show that it is necessary to use a minimum quantity of magnesia adsorbent in order to avoid loss of vitamin A.

The use of 25 ml. of 5% acetone in petroleum ether as the eluant is based on the fact that this volume of liquid will not only elute vitamin A ester from a 7 × 2.5 cm. column, but will also separate the vitamin A ester from carotene and most of the substances that repress the glycerol dichlorohydrin reaction. Under the experimental conditions, carotene is washed almost to the end of the column, but so little passes into the eluate that no correction for the reaction of glycerol dichlorohydrin with carotene need be made. Larger volumes of eluant will quantitatively elute the carotene, along with many substances which repress the glycerol dichlorohydrin reaction.

The third critical factor is the quantity of sample adsorbed. Petroleum ether extracts 2 to 6% of the solids in poultry mashes depending on the nature of the sample. From this it is evident that vitamin A in quantities usually present in mashes constitutes a small proportion of the extract. If too large a sample is used, the adsorbent will be saturated by the other components of the extract, with the result that many substances will not be retained on the column and will be eluted along with vitamin A ester. The effect of increasing the quantity of sample adsorbed on the repression of the vitamin A–glycerol dichlorohydrin reaction is shown in Table I. Forty units of standard vitamin A are added to various aliquots of a mash extract containing no vitamin A, and the samples are adsorbed and eluted in the usual manner. The data show that as the quantity of sample increases the density decreases, and indicate that the larger the sample the less the removal of substances which repress the vitamin A–glycerol dichlorohydrin. The quantity of sample adsorbed should therefore be restricted to the equivalent of 4 to 5 grams of mash. The repression factor found with a number of feeds of different composition ranged from 1.05 to 1.15 with 4- to 5-gram samples.

The procedure presented here applies only to vitamin A ester. That the free vitamin A would be strongly adsorbed (*10*) is due to the well-known effect of a free OH group on adsorption affinity (*8*). Ordinarily vitamin A is added to feeds in unsaponified fish liver oils. Because almost all the vitamin A in such oils occurs in the ester form (*3*, *5*), the method should be applicable to many fortified feedstuffs.

A number of poultry mashes were obtained with considerable variations in composition, as shown in Table II. None contained a measurable quantity of vitamin A. In order to test the recovery of vitamin A, a standard 3000A-400D feeding oil was mixed with the various feeds to give final concentrations of 500, 1500, and 3000 I.U. per pound (1.1, 3.3, and 6.6 I.U. per gram). A distilled vitamin A ester concentrate was used to give concentrations as high as 300,000 I.U. per pound. As shown in Table III, the vitamin A in most mashes can be determined within ±5% at the 3000 level and within +5 to +9% at the 1500 level.

In mash 5, the recovery of vitamin A was 108% at the 3000 level and 130% at the 1500 level. This mash was unusual, in that it contained high proportions of fish meal and distiller's solubles. The extract from this feed contained pigments that could not be completely removed, which gave a false vitamin A reaction with glycerol dichlorohydrin. Cooley *et al.* have reported similar findings, using the antimony trichloride reaction on a mash containing 20% fish meal (*2*).

The results with mashes containing 500 I.U. per pound were unsatisfactory, as the recoveries were invariably at least 20% too high. On the other hand, feeds containing more than 3000 I.U. per pound could be analyzed with an accuracy as good as or better than that found with the 3000 level. The glycerol dichlorohydrin reagent is less corrosive than the conventional antimony trichloride reagent and produces a more stable colored reaction product with vitamin A. On the other hand its use for determination of vitamin A in poultry mashes is associated with some inherent difficulties. These can be traced to the

Table II. Composition of Poultry Mashes Used in Recovery Experiments

Ingredient	Mash 1 %	Mash 2 %	Mash 3 %	Mash 4 %	Mash 5 %
White corn	17	..	...	....	30
Yellow corn	...	20	30	30	...
Ground wheat	23	20	10	12	10
Wheat bran	15	7.5	10	15	...
Ground oats	15	7.5	15	15	20
Ground barley	...	10	...	....	...
Gluten meal	...	2.5	...	5	...
Soybean meal	20	7.5	20	15	...
Alfalfa leaf meal	...	..	8	....	...
Broccoli leaf meal	...	2.5	...	1	...
Fish meal	...	10	...	5	20
Meat scraps	5	5	5	....	...
Steam bone meal	1	..	1	1	1
Whey	...	..	...	....	13
Dried skim milk	2	..	...	....	...
Molasses	...	2.5	...	....	...
Distiller's solubles	...	..	...	....	5
Delsterol	0.05	..	0.05	0.075	0.05
Salt mix	0.5	1.0	0.5	0.5	...
Oyster shell flour	1.5	3.0	1.5	1.5	0.5

Table III. Recovery of Vitamin A Added to Poultry Mashes

	500 I.U./Lb. Added		1500 I.U./Lb. Added		3000 I.U./Lb. Added		300,000 I.U./Lb. Added	
Mash	I.U./lb.	%	I.U./lb.	%	I.U./lb.	%	I.U./lb.	%
1	603	120	1590	106	2920	97	298,000	99
2	611	122	1640	109	2880	96	290,000	95.5
3	650	130	1580	105	2820	95	302,000	100.5
4	632	126	1620	108	3120	104	285,000	95.0
5	787	157	1950	130	3240	108	320,000	107

fact that some constituents of poultry mash (apparently unsaponifiable) suppress or interfere with the reaction of glycerol dichlorohydrin with vitamin A. Most of the interfering substances, but not all, could be removed by adsorption on activated magnesia Super-Cel. It was therefore necessary to rely on an increment procedure somewhat similar to that described by Oser *et al.* (*4*). However, when the true repression factor $\left(\frac{Y}{Z-X}\right)$ was applied to the data, many of the results obtained were too high. The use of an empirical factor $\left(\frac{X+Y}{Z}\right)$ resulted in fairly good recovery of vitamin A with a variety of mashes and with vitamin A concentrations as low as 1500 I.U. per pound. However, the necessity for using an empirical factor must be regarded as an undesirable feature.

Because the method is restricted to the analysis of products containing only vitamin A ester, its general application is limited. For routine or control purposes, the present method offers advantages in simplicity and ease of operation. It should not be used for samples of unknown origin.

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